Interleukin-1-receptor-associated kinase 2 (IRAK2)-mediated interleukin-1-dependent nuclear factor κ B transactivation in Saos2 cells requires the Akt/protein kinase B kinase

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The post-receptor pathway that leads to nuclear factor κ B (NF- κ B) activation begins with the assembly of a membrane-proximal complex among the interleukin 1 (IL-1) receptors and the adaptor molecules, myeloid differentiation protein 88 (MyD88), IL-1-receptor-associated kinases (IRAKs) and tumour-necrosis-factor-receptor-associated factor 6. Eventually, phosphorylation of the inhibitor of NF- κ B (I κ B) by the I κ B kinases releases NF- κ B, which translocates to the nucleus and modulates gene expression. In this paper, we report that IRAK2 and MyD88, but not IRAK1, interact physically with Akt, as demonstrated by co-immunoprecipitation and pull-down experiments. Interestingly, the association of Akt with recombinant IRAK2 is decreased by stimulation with IL-1, and is favoured by pre-treatment with phosphatase. Likewise, Akt association with IRAK2 is increased considerably by overexpression of PTEN (phosphatase and tensin homologue deleted on chromosome 10), while it is completely abrogated by overexpression of phosphoinositide-dependent protein kinase 1. These data indicate that Akt takes part in the forma-

tion of the signalling complex that conveys the signal from the IL-1 receptors to NF- κ B, a step that is much more membrane-proximal than was reported previously. We also demonstrate that Akt activity is necessary for IL-1-dependent NF- κ B transactivation, since a kinase-defective mutant of Akt impairs IRAK2- and MyD88-dependent, but not IRAK1-dependent, NF- κ B activity, as monitored by a gene reporter assay. Accordingly, IRAK2 failed to trigger inducible nitric oxide synthase and IL-1 β production in cells expressing dominant-negative Akt. However, NF- κ B binding to DNA was not affected by inhibition of Akt, indicating that Akt regulates NF- κ B at a level distinct from the dissociation of p65 from I κ B α and its translocation to the nucleus, possibly involving phosphorylation of the p65 transactivation domain.

Key words: Akt/protein kinase B kinase, interleukin-1-associated-kinase 2 (IRAK2), interleukin 1 signalling, nuclear factor κ B (NF- κ B), Saos2 osteosarcoma cell line.

INTRODUCTION

Interleukin 1 (IL-1) is a potent pro-inflammatory cytokine that has been studied extensively because of its role in inflammation and in the pathogenesis of inflammatory diseases. Its many effects include T-cell proliferation, the acute-phase response, leukocyte adhesion and migration, and an overall increase in immune reactivity, which occurs via the induction of other cytokines. Therefore IL-1 can be considered to be a link between innate and adaptive immunity. Upon binding to a transmembrane receptor, the IL-1 receptor I (IL-1RI) [1], IL-1 exerts its effects mainly through the activation of the transcription factor, nuclear factor κ B (NF- κ B), which, in turn, induces the expression of a number of cytokines and other proteins that are associated with inflammation, such as metalloproteinases, cyclo-oxygenase, nitric oxide synthase, whose genes often possess NF- κ B-binding sites in their 5'-flanking regions [2]. After a long period of minimal understanding of the mechanism of IL-1RI signalling, there has been a recent major breakthrough in our current knowledge. The activated receptor associates with an accessory protein, AcP [3], and their cytoplasmatic tails function as binding site for the

adaptor protein myeloid differentiation protein 88 (MyD88) [4]. MyD88 is recruited to the IL-1R–AcP complex through its N-terminal TIR domain, while the C-terminal death-domain couples to IL-1R-associated kinases 1 (IRAK1) [5] and IRAK2 [6]. Further downstream, the signal is relayed from the IRAKs to tumour necrosis factor (TNF)-receptor-associated factor 6 (TRAF6), a protein that is structurally and functionally related to the other members of the family of TNF receptor-activated factors, and that participates in IL-1-mediated, rather than TNF-mediated, signalling [7]. Eventually, phosphorylated IRAKs are ubiquitinated and degraded [8]. TRAF6 then transduces the signal to the kinase complex TAK1 (transforming growth factor b-activated kinase 1)–TAB1 (TAK1-binding protein), upstream regulators of the inhibitor of NF- κ B (I κ B) kinases [9]. Upon phosphorylation, I κ B releases NF- κ B, which translocates to the nucleus and modulates gene expression.

In summary, a scenario has been proposed in which the signal is relayed from one molecule to another in a linear hierarchical structure. However, several additional molecules have been involved in IL-1 signalling, such as protein kinase C ξ [10,11], Rac1 [12], Ras [13], p38 [14] and phosphoinositide

Abbreviations used: DN, dominant negative; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; GST, glutathione S-transferase; HA, haemagglutinin; HEK, human embryonic kidney; IKK, I κ B kinase; I κ B, inhibitor of NF- κ B; IL-1, interleukin 1; IL-1RI, IL-1 receptor I; iNOS, inducible nitric oxide synthase; IRAK, IL-1 receptor associated kinase; MyD88, myeloid differentiation protein 88; NF- κ B, nuclear factor κ B; PARP, poly(ADP-ribose) polymerase; PDK, phosphoinositide-dependent protein kinase; PI 3-kinase, phosphoinositide 3-kinase; λ -PPase, λ -protein phosphatase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; TNF, tumour necrosis factor; Tollip, Toll-interacting protein; TRAF, TNF-receptor-associated factor.

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3-kinase (PI 3-kinase) [15,16], which suggests that a more complex integrated circuitry might be at work. Recently, the PI 3-kinase/Akt pathway has been shown to participate to the IL-1-dependent induction of NF- κ B. However, it is unclear whether Akt affects the IL-1 receptor-dependent cytoplasmic cascade mentioned above, thereby leading to phosphorylation of I κ B [17] and nuclear translocation of NF- κ B, or contributes to the transactivation of RelA/p65 in the nucleus [18]. In this study, we confirm that Akt is activated in Saos2 cells that are exposed to physiological doses of IL-1, and that this activation is essential for IL-1-mediated NF- κ B activation. Furthermore, we demonstrate that the activation of NF- κ B, induced by the overexpression of MyD88 and IRAK2, is completely abrogated by a dominant-negative form of Akt. Moreover, for the first time, we show the physical association between Akt and each of the above molecules, pointing to the assembly of a multi-protein complex in which Akt plays an essential role.

EXPERIMENTAL

Cell culture and transfection

Human osteosarcoma Saos2 cells and human embryonic kidney epithelial (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen–Life Technologies, Groningen, The Netherlands) supplemented with 10 % fetal calf serum (EuroClone Ltd, Leeds, U.K.).

Transient transfections of 293T cells were performed by the calcium phosphate method. Saos2 cells were transfected using the FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) with a 3:1 (liposome:DNA) ratio, according to the manufacturer's instructions. After transfection, cells were serum-starved for 24 h, treated with 10 ng/ml of IL-1 β . Where indicated, cells were pre-treated for 30 min with 2 μ M LY294002 (Sigma, St. Louis, MO, U.S.A.).

Plasmids

Mammalian expression vectors containing N-terminal V5-tagged (amino-acid residues: GKPIPPLLGLDST from the P/V proteins of the Paramyxovirus SV5) human MyD88 and IRAK2 were supplied by Invitrogen. M2-IRAK1 (amino-acid sequence: DYDD-DDK) and AU1-MyD88 (amino-acid sequence: DTYRYI) were supplied by R. D'Angelo and M. Muzio, haemagglutinin (HA)-tagged wild-type Akt, its kinase-defective double mutant, myc-(phosphoinositide-dependent protein kinase 1) PDK1 and EE-tagged PTEN (phosphatase and tensin homologue deleted on chromosome 10) (amino-acid sequence EYMPME) were supplied by A. Tokar. Glutathione S-transferase (GST)-tagged IRAK2 was obtained by PCR cloning into pGEX-4T-1 (Amersham-Pharmacia, Piscataway, NJ, U.S.A.).

Luciferase reporter assay

Cells (1×10^6) were co-transfected with a 5xrel NF- κ B-Luc reporter plasmid (Stratagene, La Jolla, CA, U.S.A.) and a pRL-CMV plasmid (Promega, Madison, WI, U.S.A.) in a 1:33 ratio, together with the indicated plasmids. Luciferase activity was measured with the Firelite Dual System (Packard BioScience, Groningen, The Netherlands), following the manufacturer's instructions, and luminescence was measured with the Top Count (Packard Instruments, Meriden CT, U.S.A.). Relative NF- κ B activity was calculated by normalizing firefly luciferase activity

with *Renilla* luciferase activity. Data from experiments performed in triplicate are expressed as means $\pm$ S.E.M.

Preparation of cell extracts

Sub-confluent cells were extracted by addition of RIPA buffer (20 mM Tris/HCl, pH 7.0, 1 % Nonidet P40, 150 mM NaCl, 10 % glycerol, 10 mM EDTA, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1 mM PMSF, 10 μ g/ml leupeptin and 10 μ g/ml pepstatin) at 4 °C. Lysates were cleared by centrifugation and used for immunoprecipitation experiments, as described below. Aliquots (50 μ g) of the total lysate were boiled immediately in SDS sample buffer, resolved and immunoblotted with the indicated antibodies.

Immunoprecipitation and immunoblotting

Pre-cleared lysates were incubated for 3 h with anti-V5 antibodies (3 μ g), anti-Akt antibodies (3 μ g) or mouse IgG to provide a negative control, plus 30 μ l of 50 % (v/v) of protein A/G agarose slurry (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.) at 4 °C with gentle rocking. Pellets were washed twice with PBS containing 1 % Nonidet P40, twice in TNE (10 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA), and boiled in Laemmli sample buffer. Samples were resolved by SDS/PAGE, blotted onto nitrocellulose and probed with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies and developed with ECL (Amersham-Pharmacia).

Pull down and λ -phosphatase treatment

Recombinant His₆-HA-Akt (8 μ g) (obtained from Sf9 cells as described by Cenni et al. [19]) were incubated with 80 units of λ -protein phosphatase (λ -PPase) (New England Biolabs, Hitchin, U.K.) for 30 min at 30 °C, or with buffer alone, and added to pre-cleared lysates of 293T transfected with V5-IRAK2 or empty vector. Recombinant Akt was next pulled down with anti-HA antibody and protein A/G agarose slurry for 3 h at 4 °C with gentle rocking. Pellets were washed twice in PBS/1 % Nonidet P-40 and boiled in sample buffer.

Gel mobility and supershift assays

HEK 293 cells (10^7) were pelleted, washed in cold PBS and incubated with 400 μ l of buffer A (10 mM Hepes/KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) for 20 min, then vortex-mixed for 20 s. After centrifugation, the nuclear pellet was resuspended in 50 μ l of buffer C (20 mM Hepes/KOH, pH 7.9, 25 % glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT (dithiothreitol), 0.2 mM PMSF) for 10 min at 4 °C. Extracts were frozen in dry ice and stored at -70 °C. Nuclear extracts (10 μ g) were added to a cocktail containing 5 $\times$ binding buffer (50 mM Tris-HCl, pH 7.9, 12.5 mM MgCl₂, 5 mM EDTA, 5 mM DTT, 25 % glycerol), 0.5 μ g of poly(dI-dC) (Sigma)/reaction. γ -³²P-labelled κ B probe {obtained incubating 2 pmol of the probe with 10 μ Ci of [γ -³²P]ATP and 15 units of T4 polynucleotide kinase (Promega) for 30 min at 37 °C} was added to the cocktail and incubated for other 20 min at room temperature. Binding reactions were then run on a 5 % polyacrylamide gel in 0.25 $\times$ TBE. Competition and supershift reactions were performed by pre-incubating the nuclear extracts for 15 min at room temperature with either oligonucleotides [cold competitor, κ B oligo; uncompetitor, AP1 oligo (1 pmol each; Promega)] or polyclonal antisera specific for human p50 (H-119) and p65 (A) (2 μ g/reaction) (Santa Cruz Biotechnology).

RESULTS

Akt is activated in response to IL-1

Our previous findings demonstrate clearly a direct involvement of the lipid kinase PI 3-kinase in IL-1-dependent activation of the transcription factor NF- κ B [15,20,21]. After receptor activation by IL-1, the regulatory subunit of PI 3-kinase, p85, is recruited to the receptor, resulting in a rapid and transient increase of PI 3-kinase activity, which is essential for NF- κ B transcriptional activation, as demonstrated by the almost complete failure of IL-1 to activate NF- κ B in the presence of either specific PI 3-kinase inhibitors or dominant-negative forms of PI 3-kinase [15,20]. Moreover, mutational analysis of the receptor putative p85-binding site has revealed that the integrity of this site is essential, not only for the recruitment of PI 3-kinase, but also for transducing downstream signals to NF- κ B [15,20].

Based on several reports suggesting that PI 3-kinase effector Akt might regulate NF- κ B transcriptional activity at diverse levels [16,17,22,23], recent studies support a direct involvement for Akt in IL-1 signalling both in human endothelial cells [24], murine thymoma EL-4 6.1 and 3T3 cells [25]. Nevertheless, the evidence that after IL-1 stimulation, the activation of Akt is not required for the transcription and expression of some target genes regulated by NF- κ B [24,25] leaves the role of Akt still unclear.

To study the role of Akt in IL-1-induced inflammatory responses, we first ascertained the involvement of Akt in the IL-1RI signalling cascade in osteoblast-like Saos2 cells. To determine whether IL-1 is able to activate Akt, Saos2 cells were stimulated with IL-1 β at various times, and endogenous Akt activity was evaluated with an anti-phospho antibody that specifically recognizes the active form of the kinase [26]. Figure 1(A) shows that Akt is activated by IL-1 β , which is consistent with the proposed role of the PI 3-kinase pathway in IL-1 signalling. We then tested whether Akt activation is responsible for IL-1-induced NF- κ B transcriptional activity. Saos2 cells were transfected with a luciferase reporter driven by 5xrel sites, together with a dominant-negative form of Akt (Akt T308A/S473A). Serum-starved cells were then treated for 6 h with IL-1 β and the fold induction of luciferase activity on IL-1 β stimulation was determined. Dominant-negative Akt decreased NF- κ B transactivation by more than 50% (Figure 1B). This effect of Akt was due to transcriptional regulation and not to alterations in cell survival and cell death, since the viability of the transfected cell populations did not differ from that of cells untransfected or transfected with empty vector, as measured by anti-poly(ADP-ribose) polymerase (PARP) Western blotting (Figure 1C) and Trypan Blue exclusion (results not shown).

Akt activity is rate-limiting for IRAK2-induced, but not for IRAK1-induced, NF- κ B activation

The above results indicate that the PI 3-kinase/Akt pathway regulates the transcriptional activity of NF- κ B induced by IL-1. To get more insights, we tested the effect of the dominant-negative Akt on the downstream components of the IL-1R complex employing a clone of HEK 293 cells that were stably transfected with the receptor (293/IL-1RI). These cells behave like Saos2 cells, but represent a more suitable model for transfection experiments. The IRAK family of adaptors includes several members that share structural, as well as functional, similarities. It is known that overexpression of wild-type IRAK1 and IRAK2 is sufficient to trigger NF- κ B activation in the absence of external stimuli. Therefore, the cells were transfected with plasmids that encode these proteins, either alone or in combination with the kinase-defective form of Akt. In a dose-dependent assay, transient

Figure 1 Akt/protein kinase B kinase is activated in response to IL-1 β and regulates IL-1-dependent NF- κ B transactivation

(A) Whole extracts of Saos2 cells stimulated at the indicated times (in minutes) with 10 ng/ml of IL-1 β were immunoblotted with a specific antibody directed against phosphorylated Akt (anti-P-Akt). The blot was re-probed with an anti-Akt antibody, to confirm equal loading. (B) Saos2 cells were co-transfected with NF- κ B-luc/pRL-CMV and pcDNA3 or the dominant-negative Akt T308A/S473A (Akt DN), as indicated. After transfection (48 h), cells were stimulated with IL-1 β (grey columns) for 6 h or left untreated (NS, white columns). Relative NF- κ B activity was calculated by normalizing firefly luciferase activity with *Renilla* luciferase activity. Data from experiments performed in triplicate are expressed as means $\pm$ SEM. (C) Cells treated as above were lysed and subjected to Western blotting with anti-PARP antibody (H-250 Santa Cruz Biotech). Arrows indicate full-length PARP (112 kDa) and cleaved PARP (85 kDa). FCS, fetal calf serum. luc, luciferase.

transfection of dominant negative Akt does not affect NF- κ B activity induced by overexpression of M2-IRAK1 (Figure 2A), although a slight decrease was detectable in these cells upon IL-1 stimulation (Figure 2B), suggesting that Akt might act either upstream of IRAK1 or in a parallel pathway. On the other hand, kinase defective Akt almost completely abrogates V5-IRAK2-induced NF- κ B activity (Figure 2A), yielding results that are comparable with those observed with dominant-negative IRAK2 (results not shown). Thus, unlike IRAK1, IRAK2 depends on Akt to relay its activating signal to NF- κ B. This observation

Figure 2 Akt activity is rate-limiting for MyD88- and IRAK2-induced NF- κ B activation

(A) 293T cells were co-transfected with NF- κ B-luc/pRL-CMV and pcDNA3 (empty bars), or with NF- κ B-luc/pRL-CMV and either M2-IRAK1, V5-IRAK2 or AU1-MyD88, as specified. Dominant-negative Akt T308A/S473A (Akt DN) was added at the indicated concentrations. Relative NF- κ B activity was calculated as described above. Lysates were probed with anti-M2, anti-V5 and anti-AU1 antibodies to show equivalent expression of IRAK1, IRAK2 and MyD88, respectively. (B) 293T cells expressing the IL-1RI (293/IL-1RI) were transfected with NF- κ B-luc/pRL-CMV, alone (empty columns) or in combination with Akt DN (grey columns). After transfection (48 h), cells were stimulated with 10 ng/ml IL-1 β for 6 h or left untreated. Where indicated, cells were pre-treated with 2 μ M LY294002 for 30 min (diagonal shading). The data shown are representative of three independent experiments. CMV, cytomegalovirus; luc, luciferase.

is consistent with recent findings concerning distinct and opposite pathways originated by IRAK1 and IRAK2 [27].

MyD88 is the adaptor molecule that links the IL-1R intracellular domain with IRAK2. Interestingly, transient transfection of Akt DN (dominant negative) resulted in a dose-dependent decrease of NF- κ B activation that was dependent on AU1-MyD88 (Figure 2A).

The above results of Akt on IRAK1-, IRAK2- and MyD88-mediated NF- κ B activation were confirmed using LY294002, a specific inhibitor of the PI 3-kinase/Akt pathway (Figure 2B).

Akt physically associates with intermediates of the IL-1RI signalling complex

Collectively, the above results indicate that Akt has a functional role in IRAK2-mediated induction of NF- κ B activity. We therefore investigated whether Akt is capable of interacting with IRAK2 or other components of the IL-1R complex. HA-tagged wild-type Akt was expressed together with V5-tagged wild-type IRAK2 in serum-starved 293 cells. Immunoblotting analysis of anti-V5 immunoprecipitates showed that Akt associates with IRAK2 (Figure 3). Next, HA-Akt was co-expressed with V5-MyD88, and anti-tag immunoprecipitates were probed for the

Figure 3 Akt associates physically with IRAK2 and MyD88

293T cells were transfected with HA-tagged wild-type Akt, alone or in combination with V5-IRAK2 or V5-MyD88. Whole extracts were immunoprecipitated with anti-V5 antibodies (Invitrogen) and pellets were probed with anti-HA antibodies (Roche). The filters were then stripped and probed with anti-V5 antibody to verify equivalent levels of IRAK2 and MyD88. Equal expression of HA-Akt was monitored by immunoblotting cell lysates with anti-HA antibodies. The results are representative of three independent experiments carried out under the same conditions.

presence of Akt. Remarkably, overexpressed Akt did associate with MyD88 (Figure 3). Akt did not form a complex with the deletion mutants of MyD88 (Δ 1–152 and Δ 153–296) or with an IRAK2 mutant that lacked the death domain, IRAK2 Δ 1–96, which suggests that the association requires the integrity of both MyD88 and IRAK2 (results not shown).

The binding between Akt and IRAK1 was then analysed, but unlike IRAK2 and MyD88, the association was not detectable under either unstimulated or stimulated conditions (results not shown), which supports the notion that IRAK1 and Akt do not operate in the same pathway.

Akt affects IRAK2-dependent NF- κ B transcriptional activity

The results reported above indicate that IL-1 can regulate NF- κ B transcriptional activity through an IRAK1-dependent, but Akt-independent, pathway, as well as through an IRAK2- and Akt-dependent pathway.

To ascertain the physiological relevance of the IRAK2-dependent pathway, the expression of well-known IL-1 effectors was monitored in Saos2 cells transfected, or not, with the dominant-negative mutant of Akt. The genes for IL-1 β , inducible nitric oxide synthase (iNOS) and IL-6 are three of many that are known for being up-regulated by IL-1 during IL-1-induced inflammatory responses [24,28,29]. As shown in Figure 4(A), 8 h of exposition to IL-1 are sufficient for Saos2 cells to produce IL-1 β and iNOS. However, the expression of both proteins is decreased by dominant-negative Akt (Figure 4A) or LY294002 (results not shown). IL-6 production, in contrast, was not affected by either compound (results not shown). Furthermore, overexpression of either IRAK1 or IRAK2 was sufficient to induce IL-1 β and iNOS in the absence of stimuli. Remarkably, dominant-negative Akt impaired IL-1 β and iNOS expression promoted by IRAK2, while it was completely ineffective when the two effectors were induced by IRAK1 overexpression (Figure 4A). Using an electrophoretic mobility shift assay (EMSA), we next tested whether

Figure 4 Akt affects IRAK2-dependent NF- κ B phosphorylation and transactivation, but not DNA binding

(A) Saos2 cells were transfected with the indicated constructs, serum starved, and then treated with 10 ng/ml of IL-1 β for 8 h (or left untreated). Total cell lysates were probed with anti-iNOS (Santa Cruz Biotech) and anti-IL-1 β (R&D Systems) antibodies. Equal loading is shown by re-probing the filter with anti-actin antibodies (upper panels). Similar expression of the overexpressed proteins is shown by probing cell lysates with the respective anti-tag antibodies. Whole-cell lysates from the above experiment were probed with the anti-phospho-p65 antibody (Cell Signaling, New England Biolabs) specifically directed against the phosphorylated Ser-536. Equivalent loading is shown by re-probing the membrane with anti-p65 antibodies. (B) 293T cells were transfected with M2-IRAK1, V5-IRAK2 and HA-Akt DN as indicated, and after starvation, nuclear extracts were subjected to EMSAs. Supershifts were performed by incubating the extracts of IRAK1- and IRAK2-overexpressing cells with 2 μ g of anti-p65 or anti-p50 antibodies (Santa Cruz Biotechnology). The results shown are representative of four independent experiments. cc, cold competitor; uc: uncompetitor.

the Akt kinase deficiency might interfere with NF- κ B's DNA-binding induced by overexpression of either IRAK1 or IRAK2. Surprisingly, co-transfection of either IRAK1 or IRAK2 with a kinase-defective mutant of Akt, as well as prolonged treatment with LY294002, had no substantial effect on the ability of NF- κ B to bind a radiolabelled κ B oligonucleotide (Figure 4B), suggesting that Akt regulates NF- κ B activity at a level other than DNA binding. Moreover, overexpression of Akt DN, both alone and in combination with either IRAK1 or IRAK2, does not have any effect on the levels of I κ B α (results not shown). Collectively, these results are in agreement with previous findings by Sizemore et al. [22], and confirm that the PI 3-kinase/Akt pathway regulates NF- κ B at a level distinct from I κ B α degradation and DNA binding [22], possibly involving phosphorylation of the p65 transactivation domain [14]. In good agreement with this hypothesis, we observed that IL-1 β failed to trigger p65 phosphorylation in cells that overexpress dominant-negative Akt (Figure 4A). Remarkably, while overexpression of IRAK2 was sufficient to increase p65 phosphorylation, this effect was suppressed by dominant-negative Akt (Figure 4A).

Binding between Akt and IRAK2 is indirect and is abrogated by IL-1 stimulation

IRAKs have been shown to become highly phosphorylated upon IL-1 treatment. This conformation enables IRAKs association with other components of the receptor complex, such as MyD88, thereby allowing signal progression. However, the kinase(s) involved in phosphorylation of IRAKs is (are) not well defined. Since IRAK2 possess an Akt phosphorylation motif, IRAK2 immunoprecipitated from V5-IRAK2-expressing cells was incubated with recombinant Akt in the presence of [γ - 32 P]ATP and cofactors. Although we cannot exclude that *in vivo* conditions may lead to different results, IRAK2 is not phosphorylated by Akt *in vitro* (results not shown).

We next analysed whether the association between IRAK2 and Akt is IL-1-dependent. Total lysates of serum-starved 293/IL-1RI cells that overexpress wild-type Akt, and that were stimulated with IL-1 β at different time points, were incubated with recombinant GST-IRAK2. The beads were separated by SDS/PAGE, blotted and checked for the presence of Akt. We found that Akt associated with IRAK2 in serum-starved, untreated cells, but dissociated rapidly upon stimulation. Akt phosphorylation peaked 15 min after IL-1 β stimulation, when the binding to IRAK2 was almost absent, to return rapidly to basal level (Figure 5A). This result indicates that Akt and IRAK2 may form a complex under resting conditions, when Akt is underphosphorylated, while stimulation with IL-1 activates Akt and abolishes the interaction. Based on these results, we asked whether the binding of IRAK2 and Akt is phosphorylation-dependent. Recombinant Akt was purified from Sf9 cells that were infected with a baculovirus driven His $_6$ -HA-Akt plasmid. Purified His $_6$ -HA-Akt was pre-incubated with λ -PPase or with buffer alone, then incubated with lysates from 293T cells that expressing V5-IRAK2. As expected, purified Akt was able to pull down V5-IRAK2 from cell lysates. However, this association was increased by treatment of Akt with phosphatase (Figure 5B), which suggests that Akt phosphorylation plays a key role in its dissociation from IRAK2. We then tried to modulate Akt phosphorylation *in vivo* by transient transfection of IRAK2 with either PDK1 kinase, which specifically phosphorylates Akt at Thr 308 , or PTEN, a dual-specificity phosphatase that dephosphorylates phosphatidylinositol 3,4,5-trisphosphate at the 3'-OH position. Compared with control cells transfected with empty vector, overexpression of PTEN increased the association of endogenous Akt with IRAK2. Western blotting of cell lysates with

Figure 5 Inactivation of Akt up-regulates its binding to IRAK2

(A) Upper panels: 293/IL-1RI transfected with HA-Akt, starved and stimulated at the indicated times (min) with IL-1 β , were lysed and then incubated for 1 h with 8 μ g of GST-IRAK2 conjugated with glutathione-sepharose beads. The complexes were then revealed with anti-HA antibody to reveal the presence of Akt (arrow). The filter was reprobed with an anti-GST antibody to verify equal amounts of GST-IRAK2. Lower panels: the Akt phosphorylation pattern in response to IL1 was checked by an anti-phospho-Akt (Ser⁴⁷³) antibody. Equivalent expression of HA-Akt is also shown. (B) Lysates from 293 cells transfected with V5-IRAK2 or empty vector were incubated with His₆-HA-Akt, pretreated or not with λ -PPase, and subjected to immunoprecipitation with an anti-HA antibody. Pellets were resolved by SDS/PAGE and immunoblotted with anti-V5 and anti-HA antibodies. Cell lysates (50 μ g) were immunoblotted with anti-phospho-Akt antibody to confirm λ -PPase activity. Equivalent expression of V5-IRAK2 in total cell lysates is also shown. (C) Upper panels: lysates from 293/IL-1RI cells, transfected as indicated, were immunoprecipitated with an anti-Akt antibody and the pellets were probed with an anti-V5 antibody. Equivalent levels of immunoprecipitated Akt are shown. Lower panels: equivalent expression of V5-IRAK2, Akt, Myc-PDK1 and EE-PTEN were monitored by probing the extracts with anti-V5, anti-P-Akt, anti-Akt, anti-myc or anti-EE respectively. The results shown are representative of three independent experiments.

anti-phospho-Akt, which specifically recognizes the phosphorylated Ser⁴⁷³, reveals that, as expected, in the presence of overexpressed PTEN, Akt is underphosphorylated. On the other hand, the association was completely abrogated by ectopically expressed PDK1 (Figure 5C).

Taken together, the above results suggest that Akt recruitment by IRAK2 is regulated by Akt phosphorylation. This last consideration prompted us to explore the nature of the interaction between Akt and IRAK2 further. However, the lack of interaction between recombinant His₆-HA-Akt and GST-IRAK2, as evaluated by an overlay assay, suggested that the association between the two proteins is not direct (results not shown).

Endogenous Akt co-immunoprecipitates with IRAK2 and IL-1RI in an IL-1 β -dependent manner

To confirm that IL-1-mediated activation of endogenous Akt is responsible for the formation of the complex with IRAK2, serum-

starved 293/IL-1RI cells, transfected with wild-type IRAK2, were treated with 10 ng/ml of IL-1 β at different time points, and endogenous Akt was immunoprecipitated from whole-cell lysates. The immunoprecipitates were checked for the presence of IRAK2. In agreement with the above results, Figure 6 shows that the association between IRAK2 and endogenous Akt is readily detectable under unstimulated condition, but is almost suppressed by IL-1 β stimulation. Moreover, pretreatment of cells with the Akt inhibitor LY294002 resulted in an enhanced binding between Akt and IRAK2. Taken together, these results confirm that Akt phosphorylation modulates its binding to IRAK2.

Having demonstrated that Akt can form a complex with IRAK2, we investigated whether Akt can form a complex with IL-1RI. Endogenous Akt was therefore immunoprecipitated and checked for the presence of the receptor. Figure 6 shows that the binding was very weak in unstimulated cells, but became rapidly detectable upon activation of Akt by IL-1 β (as confirmed by the anti-phospho-Akt analysis). The last result shows that, upon activation,

Figure 6 IL-1 differentially regulates the binding of endogenous Akt to IRAK2 and to IL-1RI

Upper panels: 293/IL-1RI cells transfected with V5-IRAK2 were treated with 10 ng/ml of IL-1 β or left untreated. Lysates were incubated with an anti-Akt antibody. Where indicated, cells were pre-treated with 2 μ M LY294002 for 30 min. Pellets were immunoblotted with anti-V5 or anti-IL-1RI antibodies. Equal amount of immunoprecipitated Akt was confirmed by immunoblotting. The results shown are representative of three independent experiments.

Akt moves from IRAK2 to the receptor, indicating that IL-1 regulates the association of Akt with IRAK2 and with the receptor in different ways.

DISCUSSION

Over time, dissection of IL-1 signalling has proved difficult and controversial. A consensus has been reached recently on the IL-1-dependent assembly of a multi-protein membrane-proximal signalling complex to which the adapter molecules MyD88, IRAKs and TRAF6 are recruited in turn. As a result, the signal is relayed from one member to the following, culminating in the activation of NF- κ B. However, several points are still unclear and it is likely that additional proteins are implicated in this process.

We and others have shown previously that PI 3-kinase is involved in IL-1-mediated activation of NF- κ B [15,16,22]. More recently, a well-known PI 3-kinase effector, the serine/threonine kinase Akt, has been proposed to regulate NF- κ B transcriptional activity, both by IL-1 [22] and TNF [17]. Nevertheless, the molecular mechanisms underlying this event remain contentious. It has been claimed that Akt triggers NF- κ B by directly phosphorylating I κ B kinase α (IKK α) at Thr²³ [17]; however, these results have been challenged by reports that suggest that only IKK β , and not IKK α , is required for I κ B degradation and NF- κ B activation *in vivo* [30–32]. Moreover, Thr²³ on IKK α does not seem to be phosphorylated *in vivo* upon TNF stimulation of 293 cells [33].

In this paper, we present evidence that Akt becomes phosphorylated, and is therefore active, upon IL-1 β stimulation of Saos2 cells (Figure 1A). In agreement with other reports, Akt activity is necessary to trigger IL-1-dependent NF- κ B activation, as overexpression of a kinase defective form of Akt abrogates the response to IL-1 in these cells almost completely (Figure 1B). For the first time, we demonstrate that this effect originates at a membrane-proximal level, where Akt interacts with members of the receptor complex, namely MyD88, IRAK2 and the IL-1RI itself. Indeed, our results show that a kinase-defective mutant of Akt completely prevents transcriptional activation of NF- κ B due to overexpression of MyD88 and IRAK2, which indicates that Akt operates in the same pathway as these molecules. However, quite unexpectedly, dominant-negative Akt failed to suppress IRAK1-induced NF- κ B activity. In fact, overexpression of wild-type IRAK1 is sufficient to trigger NF- κ B transactivation to the same extent both in the presence and in the absence of a kinase-

defective form of Akt. Intriguingly, dominant-negative Akt and the PI 3-kinase/Akt kinase inhibitor LY294002 were able to reduce slightly IRAK1-dependent NF- κ B activation, only after exposing the cells to physiological doses of IL-1, which suggests that Akt acts upstream of IRAK1 or in a parallel pathway.

Three members of the IRAK family, namely IRAK1, IRAK2 and IRAKM (IRAK-like molecule) share high structural and functional homology, operate downstream of MyD88 in the NF- κ B activatory cascade, and can restore the response to IL-1 in an IRAK1-deficient 293 cell line [34]. A distinct feature among the IRAKs is the intrinsic kinase activity, which seems to be a prerogative of IRAK1. This implies that each member of the family might differentially influence cellular signalling processes. Indeed, IRAK1 has been shown to phosphorylate Tollip (Toll-interacting protein), leading to dissociation of Tollip from the receptor complex [35]. Interestingly, IRAK1 and IRAK2 have been shown recently to exert opposite effects on macrophage survival: in YopP-transfected macrophages stimulated with lipopolysaccharide, IRAK2 relays a pro-apoptotic signal, while IRAK1 is involved in the NF- κ B anti-apoptotic cascade [27]. Moreover, a novel member of the family, namely IRAK4, which has been cloned recently [36,37], shares the domain structure of the other members and functions in the same signalling pathways. IRAK4 is an active kinase and its activity is required upstream of IRAK1 to activate NF- κ B, possibly by direct phosphorylation of IRAK1 in response to IL-1, but it is not able to restore the sensitivity to IL-1 in the IRAK1-deficient 293 cell line [37]. These distinct features of the IRAK family members suggest that their function is not redundant. Our observation that kinase-inactive Akt specifically impairs IRAK2-dependent, but not IRAK1-dependent, NF- κ B-mediated expression of specific genes, namely those that code for iNOS and IL-1 β (Figure 4A), substantiates the above model, and helps to explain how the specificity of each response is reached. With respect to IL-6, our observation that dominant-negative Akt failed to prevent both IRAK1- and IRAK2-dependent production of this cytokine is in agreement with a recent observation by Neumann et al. [25], who reported that wortmannin fails to block IL-1-stimulated IL-6 production. Our study also shows that IRAK2 and MyD88, but not IRAK1, interact physically with Akt, as demonstrated by co-immunoprecipitation experiments (Figure 3), which indicates that Akt takes part in the formation of the signalling complex, which conveys the signal from the IL-1Rs to NF- κ B downstream activators. This hypothesis is supported further by the observation that the integrity of both MyD88 and IRAK2 is crucial for their interaction with endogenous Akt, as removal of either the N- or the C-terminal domains prevents aggregation of both an Akt–MyD88 and an Akt–IRAK2 complex (V. Cenni and S. Marmioli, unpublished work). Interestingly, the association of Akt with recombinant IRAK2 is decreased by IL-1 stimulation (Figure 5A), and is favoured by pre-treatment with phosphatase (Figure 5B). Moreover, consistent with its role as Akt antagonist, overexpression of PTEN increases Akt association with IRAK2 considerably. On the contrary, the recruitment is completely abrogated by overexpression of PDK1, the upstream Akt activator (Figure 5C). Collectively, these results imply a mechanism whereby the recruitment of IRAK2 depends on the activation state of Akt. Recently, works by Burns et al. [38] and by Zhang and Ghosh [35] revealed that the adaptor protein Tollip forms a complex with IRAKs in resting cells and inhibits signalling by blocking IRAKs phosphorylation that occurs when, following IL-1 stimulation, Tollip is phosphorylated. According to Zhang and Ghosh [35], and consistent with the existence of a highly conserved UBA (ubiquitin-associated) and CUE (coupling of ubiquitin conjugation to the endoplasmic reticulum) domains

at its C-terminus, Tollip might undergo degradation. On this basis, it is tempting to speculate that, subsequent to IL-1 stimulation, activated Akt might phosphorylate and therefore inactivate an adaptor molecule, such as Tollip itself or similar to Tollip, that entraps IRAK2 in a conformation inaccessible to MyD88, leading to IRAK2 release. On the other hand, a kinase defective version of Akt would interfere with IL-1 signalling by retaining IRAK2 in a complex, thus reducing the effect of IL-1 on NF- κ B activation. On this basis, we performed a Scansite search (<http://scansite.mit.edu>) that revealed that human Tollip possesses a good putative phosphorylation site for Akt at its C-terminus. However, an *in vitro* kinase assay to test the ability of Akt to phosphorylate Tollip revealed that Akt is not a Tollip kinase (V. Cenni and S. Marmioli, unpublished work). This indicates that either Tollip interaction with IRAK2 is regulated by mechanisms other than Akt phosphorylation, or that an unidentified protein might be responsible for sequestering IRAK2. Recently, Akt was found to be directly involved in the phosphorylation of IRAK1 [39]. According to Chen et al. [39], IL-1 induced CaMKK-mediated Akt activation increases IRAK1 phosphorylation, leading to the uncoupling of IRAK1 from MyD88 and the inhibition of NF- κ B activity [39]. Furthermore, and in conflict with our and previously reported results, neither LY294002 nor dominant-negative Akt show any effect on IL-1 β -regulated NF- κ B activity in the above model. We do not have an explanation for this discrepancy at present. However, it should be noted that the negative effect of Akt described by Chen et al. [39] is a late event, as it was monitored after 24 h of treatment with IL-1 in HEK 293 cells, which express very low levels of IL-1RI, while the results presented here were obtained after 2–6 h treatment of cells expressing the IL-1RI.

In addition, our results confirm the role proposed for Akt in the regulation of NF- κ B activity; however, in our model the function of Akt is restricted to an IRAK2-dependent pathway. In fact, both dominant-negative forms of Akt and LY294002 are absolutely ineffective on IRAK1-induced NF- κ B transactivation. Conversely, our results demonstrate clearly that NF- κ B transcriptional activity induced by overexpression of IRAK2 is severely decreased when Akt activity is abrogated by transfection with dominant-negative forms of Akt or by LY294002, as detected by a gene reporter assay. Accordingly, NF- κ B-dependent expression of iNOS and IL-1 β triggered by IRAK2 is significantly decreased by dominant-negative Akt. Despite the clear effect on NF- κ B transactivation, our results demonstrate that neither NF- κ B binding to DNA (Figure 4B) nor I κ B α degradation (results not shown) are affected by the PI 3-kinase/Akt pathway, since both LY294002 and dominant-negative Akt are unable to reduce the above effects, as detected by EMSA. Collectively, these data indicate that Akt regulates NF- κ B transactivation, and not NF- κ B nuclear translocation and DNA binding, possibly through phosphorylation of the transactivation domain of p65. In this respect, it is worth noting that dominant-negative Akt abrogates p65 phosphorylation triggered both by IL-1 and IRAK2 (Figure 4A). These observations are in good agreement with the data reported by Sizemore et al. [22], demonstrating that Akt operates in a pathway that is necessary for the transactivation of NF- κ B, but apart from that, that leads to I κ B degradation and NF- κ B binding to DNA [14,40,41].

In conclusion, the results presented here corroborate the previously reported role of Akt in NF- κ B regulation, and add new insights showing that Akt can act on NF- κ B through an IRAK2-mediated pathway. In addition, we demonstrate that Akt can operate in this pathway at a more membrane-proximal level than was hypothesized previously, given that it can form complexes with MyD88, IRAK2 and IL-1RI, and that IL-1 β differentially

regulates these interactions. Taken together, these results point to a novel, membrane-proximal function of Akt in the NF- κ B regulatory pathway and indicate IRAK2 as a bifurcation point in NF- κ B regulation.

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